Remarks

Claims 1-29 and 31-41 are pending. Claims 29 and 41 has been amended. Claim 30 has been canceled.

The present invention is related to the finding that human tumor cells can be killed by the administration of a prodrug, CB 1954, and the exogenous co-substrate, reduced nicotinamide riboside or "NRH" or an analog thereof, for an enzyme, human NAD(P)H:quinone reductase 2 "NQO2". The prior art describes the administration of the prodrug, CB 1954, in combination with an enzyme, to kill tumor cells. See, for example, U.S. Patent No. 5,780,585, and U.S. Patent 5,958,682, which teach the use of an *E. coli* nitroreductase enzyme to activate CB 1954 for the treatment of human tumors. In contrast to the prior art which discloses the administration of an enzyme with the prodrug, the applicants have discovered one can administer a prodrug along with an exogenous co-substrate for an enzyme, NQO2, which in combination with the co-substrate is able to convert the prodrug into a powerful cytotoxic compound.

Experiments in animals harboring tumors have shown that when a prodrug is specifically activated in the tumor environment, the animal is often cured of the cancer. The specificity of the treatment lies at the core of the efficacy of the treatment. The delivery of a cytotoxic agent specifically to tumor cells is highly desired. Normal cells are often killed when cytotoxic agents lack specific targeting or are administered systemically. There are may examples of using prodrugs which are selectively activated by enzymes present in tumors. The most familiar example is gangciclovir, which is activated in the presence of thymidine kinase to yield a cytotoxic compound. Assuming that the prodrug is a good substrate for the enzyme specifically

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants: Philip John Burke and Richard John Knox

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For: *"THERAPEUTIC SYSTEMS"*

Commissioner of Patents and Trademarks

Washington, D.C. 20231

DECLARATION UNDER 37 C.F.R. §1.132

Sir:

I, Professor Richard John Knox, hereby declare that:

1. I have a PhD in biochemistry and have 24 years experience in cancer research. Until I took up my present position I was Team Leader of the Molecular Pharmacology Unit, Section of Drug Development (now the Cancer Research UK, Centre of Cancer Therapeutics) at the Institute of Cancer Research, Sutton, England. I am a member of both the European Organisation for Research and Treatment of Cancer (EORTC) screening and pharmacology group committee and the pharmacology and molecular mechanisms committee. I have also served on the CRC Targeting Trials Committee. My present position is Director of Molecular Pharmacology at Enact Pharma plc and I am also Visiting Professor in Molecular Pharmacology at the University of Greenwich, England. I have worked on enzyme/prodrug strategies since 1986. I have worked in the field of cancer therapy using enzyme/prodrug strategies for a total of 16 years and have been responsible for the design and synthesis of prodrugs, elucidating the background science of such strategies and the clinical development of components. I am an inventor on granted US patents (nos. 5,780,585 and 5,958,682) demonstrating means of activating CB 1954 in *human* tumours.

2. I have been working with CB 1954 for a number of years and was responsible for elucidating the mechanism of action of CB 1954 as an anti-cancer agent and the precise reason why human tumour cells are normally insensitive to CB 1954.

3. I am an inventor on US patent application no. 10/099,830, continuation of 09/445,865 (hereinafter referred to as "the patent application"). The patent application contains two examples (Examples 7 and 8) which demonstrate the cytotoxicty of CB 1954 to cells in the presence of an exogenous co-substrate and NQO2 *in vitro*. Example 7 uses V79 cells that have been transfected with NQO2. In Example 8 in the patent application, I used non-transfected human tumour T98G cells, which comprise NQO2 naturally, to show the *in vitro* activity of the combination of CB 1954 with NRH and analogues on human tumour cells. Other examples of cell types have been used and the results published [**Knox R.J.,** Jenkins, T.C., Hobbs, S.M., Chen, S., Melton, R.G. and **Burke, P.J.**, *Cancer Research*, 2000, 60, 4179-4186].

4. It is generally accepted in the art that results from *in vitro* and *in vivo* experiments may be usefully extrapolated for the evaluation of treatments for human tumours. Thus, the T98G cells which we have used in our experiments have been used by other workers, both *in vitro* and *in vivo,* for evaluating anticancer agents [see, for example, Nanda, D., Vogels, R., Havenga, M., Avezaat, C.J., Bout, A. & Smitt, P.S. (2001) *Cancer Res*, **61,** 8743-50.; Rubenstein, M., Shaw, M., Mirochnik, Y., Slobodskoy, L., Glick, R., Lichtor, T., Chou, P. & Guinan, P. (1999). *Methods Find Exp Clin Pharmacol*, **21,** 391-3; Teicher, B.A., Alvarez, E., Mendelsohn, L.G., Ara, G., Menon, K. & Ways, D.K. (1999) *Adv Enzyme Regul*, **39,** 313-27;

Yang, J., Yang, J.M., Iannone, M., Shih, W.J., Lin, Y. & Hait, W.N. (2001) *Cancer Res*, **61,** 4010-6)]. It is apparent from these studies that the authors are using *in vitro* activity as an indication of *in vivo* activity of potential anticancer agents using T98G cells (see Teicher *et al*, 1999 and Yang *et al*, 2001). Similar arguments have been made for the other cell lines and for the xenografts that we have used [see, for example, Teicher, B.A., Schwartz, G.N., Dupuis, N.P., Kusomoto, T., Liu, M., Liu, F. & Northey, D. (1994). Oxygenation of human tumor xenografts in nude mice by a perfluorochemical emulsion and carbogen breathing. *Artif Cells Blood Substit Immobil Biotechnol*, **22,** 1369-75 and Radulovic, S., Miller, G. & Schally, A.V. (1991). Inhibition of growth of HT-29 human colon cancer xenografts in nude mice by treatment with bombesin/gastrin releasing peptide antagonist (RC-3095). *Cancer Res*, **51,** 6006-9].

5. I have reviewed the Office Action mailed 2 October 2001 in which Claims 29, 31-33, 40 and 41 are rejected under 35 U.S.C § 112 as failing to provide sufficient guidance and/or objective evidence that the method would predictably treat a patient with cancer.

6. I believe that the administration of CB 1954 and a specific co-substrate would reasonably be expected to be useful for the treatment of cancer in a human patient on the basis of the results that are given in the patent application, for at least the reasons that are set out below.

7. Certain rat cells contain the enzyme NQO1 (NAD(P)H quinone oxidoreductase (DT-diaphorase)), which can effectively activate CB 1954 *in vivo*. CB 1954 therefore exhibits a dramatic and highly selective activity against the rat Walker 256 tumour and actually cured this tumour [see, for example, Cobb, L.M., Connors, T.A., Elson, L.A., Khan, A.H., Mitchley, B.C., Ross, W.C. & Whisson, M.E. (1969), *Biochem Pharmacol*, **18,** 1519-27 and Connors, T.A. & Melzack, D.H. (1971), *Int J Cancer*, **7,** 86-92]. Rat NQO1, in the presence of NADH or NADPH, catalyses the aerobic reduction of CB 1954 to its 4-hydroxylamino derivative. The 4-hydroxylamino derivative of CB 1954 is highly cytotoxic and it is the formation of this derivative *in vivo* or *in vitro* which is believed to account for the effectiveness of CB 1954 against a range of cell types, including human tumour cell lines. Thus, Walker cells are not uniquely sensitive to the cytotoxic derivative of CB 1954, which can also be effective against other tumour cells [Boland M.P., **Knox R.J.**, and Roberts J.J. *Biochem Pharmacol*, 1991, 41, 867-875].

8. The human equivalent to rat NQO1, human NQO1, is present in raised levels in a number of human cancers such as colon and liver. However, human NQO1 metabolises CB 1954 much less effectively than rat NQO1. Because human NQO1 is intrinsically less able to produce the cytotoxic 4-hydroxylamino derivative of CB 1954, human cells appear to be resistant to CB 1954. It is, however, accepted that the introduction of an enzyme into human tumour

38. Use of a prodrug which is converted to a substantially cytotoxic drug by the action of NQO2 in the manufacture of a medicament for treating a human patient with a target cell to be destroyed wherein the patient has been, is being or will be administered NRH or an analogue thereof which can pass reducing equivalents to NQO2.

39. A kit of parts comprising a means for determining whether a target cell to be treated expresses NQO2 and NRH or an analogue thereof which can pass reducing equivalents to NQO2.

40. The method of claim 29 wherein the patient has cancer.

41. (New) The method of claim 29, wherein the analogue of NRH is 1-(carboxamidomethyl)-dihydronicotinamide.

and **Burke, P.J.**, *Cancer Research*, 2000, 60, 4179-4186]. Importantly, we have found that NQO2 is only activated towards CB 1954 in the presence of certain *exogenous* non-biogenic co-substrates, such as nicotinamide riboside (NRH) and analogues thereof. Thus, human NQO2 in combination with an exogenous co-substrate is about 3000 times more effective than human NQO1 and endogenous co-substrates in the reduction of CB 1954 to its highly cytotoxic 4-hydroxylamino derivative.

11. A number of human tumour cell lines have an increased sensitivity towards CB 1954 in combination with NRH and contain endogenous NQO2 that can be measured using a biochemical assay. There is no reason to suppose that CB 1954, activated using our method, should be less effective as an anti-tumour agent than CB 1954 activated by any other method (for example, as in rats where it is a proven anti-tumour agent).

12. The following *in vivo* experiments show that when a combination of a specific co-substrate and CB 1954 are administered to human cells, human NQO2 together with the co-substrate efficiently converts CB 1954 to its cytotoxic form, which inhibits the growth of tumours.

13. Experimental therapy of four types of human tumour xenografts has been performed under my direction. A xenograft is a tissue graft where the donor is a different species to the

recipient. In experimental cancer chemotherapy the tissue is normally a human cancer that is grown on a mouse. This allows the effect of drugs to be assessed on human cancer cells in a whole body situation. Evaluation of drugs *in vivo* is important because it allows the effect of drug pharmacology, metabolism and toxicity to be accessed. In the case where the HT-29 and BE colon derived xenografts were used, CB 1954 was administered I.P. at the maximum tolerated dose of 80mg/Kg followed 1hr later with 200mg/Kg of NRH administered I.V.. This protocol was not optimised. However, a substantial growth delay was observed and this was superior to that achievable using the established anti-tumour agent Cisplatin (see Table 1).

Table 1. Therapy of Human HT 29 or Be Tumour Xenografts

	HT 29		**BE**	
AGENT	**T/C**	**G.D.**	**T/C**	**G.D.**
CONTROL	1	0	1	0
NRH (200mg/kg I.V.)	0.982	-0.4	0.968	0.3
CB 1954 (80mg/kg I.P.)	0.358	21.9	0.566	7.2
CB 1954 + NRH	0.251	33.2	0.313	17.4
CISPLATIN (MTD, 4mg/kg)	0.582	8.9	0.427	7.3

T/C Ratio of tumour volume to control at 28 days

G.D. Growth delay

MTD Maximum tolerated dose

The xenograft therapy studies of HT-29 and BE tumours were performed at the Institute of Cancer Research, Sutton, Surrey.

14. In two further studies, therapy of the PC-3 (prostate derived) or LoVo (colon derived) human tumour xenograft was achieved using a daily (x4) dosing schedule of 25mg/Kg CB 1954 followed by either 250mg/Kg of NRH (PC-3) or a 1-carboxamido derivative of dihydronicotinamide, EP-0152R, (PC-3 and LoVo). In the presence of either co-substrate, CB 1954 again showed a very substantial growth delay with EP-0152R giving an even better therapy than NRH (see Figure 1 in Appendix). Neither CB 1954 alone nor co-substrate alone showed any significant anti-tumour activity (see Figures 1 and 2 in Appendix).

15. These studies on tumour xenografts, that is to say carried out *in vivo*, confirm what I

expected at the time I filed my patent application. Thus, CB 1954 can be activated by NQO2 in the presence of a co-substrate to give a cytotoxic effect in living systems. The discovery of the ability of the enzyme NQO2 to efficiently activate CB 1954 in human tumour cells in the presence of a specific *exogenous* co-substrate (NRH or analogs thereof) *in vitro* and *in vivo* allows a human enzyme that may be endogenous to certain cells in the body to be exploited.

16. From my experience, the disclosure in the patent application enables an effective therapy for the treatment of tumours in humans. In the patent application, the feasibility of using the prodrug CB 1954 in combination with an appropriate co-substrate to kill human cancer cells is demonstrated. A person skilled in the art would recognise that treatment of cancer in humans could be achieved using this combination of compounds. I have also carried out the further *in vivo* testing as mentioned above and have achieved a significant anti-tumour effect against human tumour xenografts. Such *in vivo* testing would give the investigator the confidence that the proposed treatment would be both effective and safe for the treatment of humans.

17. It is my belief that one skilled in the art would know how to treat tumours in humans using CB 1954 based on the description in the patent application as filed, with a reasonable expectation of success.

I declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true, and further, that these statements are made with the knowledge that willful false statements are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Date: 7th August 2002 R J K

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APPENDIX

Figure 1. Therapy of PC-3 Human Prostate Tumour Xenografts. CB 1954 (25mg/Kg) was given I.P. daily (x4) followed 30 minutes later by either 250mg/Kg of NRH or EP-0152R given I.V.